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COMMUNICATIONS

NEW ULTRAMICRO METHODS IN BIOCHEMISTRY

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Ever since work has begun with biochemistry, people have always attempted to work with ever smaller quantities and the ultimate aim of this development is certainly the quantitative determination of the chemical constituents of an isolated cell, or of a small group of identical cells. We are still very far from that aim, but great strides have already been made in that direction. It is evident that in the course of this development the instrumentation which will be used will undergo deep modifications in order to make it possible to handle minute quantities with a precision that will still be sufficient. On the other hand, it is evident that new methods will be sought in the physical chemistry and organic chemistry domains that will permit precise and specific dosages. Two sciences which have, until now, been quite separate from each other, meet in this matter and can facilitate each other's task: those are histochemistry, which works solely with specific and qualitative methods, and analytical chemistry, which used precise processes, which, however, are often less specific. The problem is immense and I shall try to tackle only one small sector of it, viz., the problem of equipment and methods in analytical chemistry, which makes it possible to work easily with considerably reduced volumes of biological liquid.

In this realm, which is now called "ultramicro" or "submicro", the pioneer is certainly Linderstrom-Lang, whose works in the realm of cellular chemistry have become a classic. Other researchers have followed him, especially Kirk in analytical chemistry, Quick in semi-quantitative histochemistry and Sobel in biological chemistry.

A very great number of methods and instruments have been described in a wide variety of periodicals. But with the exception of electrophoresis on paper and of photometry of the flame, the ultramicro methods have not achieved a general usage, for they require an extremely delicate instrumentation and a

specialized personnel. We have developed a general equipment which renders possible a very easy and rapid work with samples of the order of 10 (1/100 of a ml), which are very convenient for routine analyses. This equipment comprises tubes for the collection and centrifugation of biological liquids, of automatic pippettes to measure the samples and the reagents, stirring sticks, titration tables, cruets, special electrodes, diffusion units, new optical vats and various accessories permitting the dosage of most biological components.

Without entering into the detail of the various dosages, I intend to discuss here some basic operations which are identical for a very great number of determinations.

Collection and centrifugation of the sample : a simple polyethylene tube of 2 to 3 mm of diameter and 12 to 14 cm long serves well for taking samples of all biological liquids, including capillary blood and tissue extracts. For transport and centrifugation the tube is bent in two. It is placed directly in a centrifuge without the need of an equilibration, since its weight is small. The tubes are bought pre-cut and are ready for the use without any preliminary washing, and the price of about 1 Swiss centime per piece. Volumes of 0.1 to 1 ml are very rapidly centrifugated (centrifugalized), since these tubes bear centrifugation speeds up to 10,000 turns per minute. All the usual centrifuge models can be used.

After centrifugation the tube is cut at the desired level in order to make easier the taking of an aliquot, then is thrown away.

The advantage of these tubes on glass tubes are the following: no cleaning, no losses through breakage, no equilibration before centrifugation.

The pipetting of the aliquot must be made with the greatest possible precision. The precision of the final result depends largely on this operation.

We use polyethylene (fig. 1) and always the same pipette for the same dosage: thus one pipette for chlore, one for the urea, one for the proteins, etc.

The various samples in which the same component has to be dosed are taken with the same pipette, as well as the standard solution, or the standard serum which we have introduced in our experiences for each dosage series. Thus, we no longer need to know exactly the volume of the pipette and it will not be the reproductibility of the volume taken as sample which will determine the precision of the sampling. The reproductibility is very good (typical deviation with human serum $s = \pm \sqrt{\frac{\sum (\bar{x} - x_i)^2}{n-1}}$ n = 20. For 5 μl : s = $\pm$0.41

p. 100; for 10 μl : s = $\pm$0.39 p. 100; for 20 μl : s = $\pm$0.17 p. 100. It is much better than with glass pipettes, where it varies between $\pm$1 to 4 p. 100 of the volume for volumes of 0.1 ml, by using a different pipette for each measurement.

In filling the pipette, care will be taken to always allow a little drop to flow out at the upper end, thus insuring a washing of the pipette by the liquid itself, and allowing a perfect reproduction of the volume included in the capillary tube. In order to insure a minimum of precision one must empty the pipette slowly. Thus, we manage to reproduce volumes between 0.1 and 1000 μl with a hitherto unknown precision. We take with the pipette one sample after another without ever washing the pipette. The volume of a sample remaining in the pipette and mixing with the next one is less than 1 per 10,000, as we were able to prove it with proteins marked with radioactive iodine.

One final remark remains to be made: during the titration of protein solutions a very fine layer of proteins settles on the sides, changing volume in the long run, and influencing the evacuation. That is also true for glass pipettes. This protein film is difficult to dissolve and to digest. For this reason, after a day of work, a pepsin or papain solution should be kept in the pipette, which it completely regenerates.

In our reagent pipettes, the same kind of polyethylene tube (fig. 1) is mounted in the glass tube which communicates on the one hand with the outside by a lateral tube and with the inside, on the other hand, by means of a little polyethylene flask which contains the reagent.

By a very simple manipulation (two successive pressures on the flask) the capillary is at first filled with reagent and then emptied with a reproductibility still a little above that of the sample pipette. (Typical deviations for 2.5 μl = $\pm$0.128, for 20 μl = $\pm$1.124 p. 100). The volumes which are delivered can vary between 0.1 and 1000 μl (1/10,000 - 1 ml) and here, too, the exact volume is not important. The reagent is always ready to be used and is protected from the outside; the flask contains enough reagent for 500 to 1500 analyses and there is no need to wash the pipettes or the test-tubes. Each reagent for each dosage is in its bottle and the person conducting the experiment can never make a mistake in the matter of volume. A similar set-up, entirely made of glass surrounded with a polyethylene flask and having an identical functioning is used for reagents or solvents which react with polyethylene.

The automatic functioning of both types of pipettes renders possible a very easy work. The fact that they are inacessible is also a great advantage.

For centrifugalization there is advantage to use a small centrifuge of up to 20,000 turns per minute with sloping tubes, of 0.1 to 1 ml, needing no other equilibration.

All filtration -- with the exception of ultrafiltration under pressure -- must be avoided on the ultramicro scale.

The three main problems in the titration of small volumes are: the test-tube, the agitation and the determination of the point of veering.

We have developed a test-tube (fig. 2) which makes possible the direct reading of volumes between 0.03 to 90 μl with a precision of $\pm$ 0.01 μl. The volumes which are usually employed for the titration are of 5 to 10 μl. They are read on a big dial with needle, with an automatic reduction to zero. The total capacity of the test-tube is 150 μl (0.15 ml).

The liquid is delivered by means of a fine point in polyethylene which dips into the solution that must undergo titration.

The agitation is carried out with the aid of a stirring stick which is vibrating and which is moved by an aquarium with the aid of a "boa" cable (flexible, but rigid in each position). The movements of the stirring stick with the polyethylene point are sinusoidal in shape and has a frequency of 50 per second, which insures excellent stirring, while avoiding any loss through spraying.

But the determination of the veering point may be achieved by using the usual indicators. However, it is advantageous to take advantage of the greatest sensitivity and precision of physical measurements such as potentiometry (for example, a glass microelectrode shaped like a cupule), conductometry, photometry (photometric titration with photoelectric microcells), polarimetry, etc.

Photometry, which gives a reading of light absorption by small volumes of colored solutions, requires a good photometer (with filters or a spectrometer) equipped with microcurves. We have shown a stationary curve in a small photometer with filter with a very fine light ray (Beckman Mod. C.). 0.25 ml of colored solution is enough for one reading. By pressing on a button, the curve empties automatically, the liquid being sucked into a waste bottle by a small suction pump. More than 99.5 per cent of the liquid is taken out by this suction. Thus, the measurements of samples can come one after another without any washing in between, and with great speed and precision.

After each twentieth reading the vat is rinsed with a detergent solution (oronox), with water, then treated with a 1 per cent solution of N-Octanol in acetone, and is washed anew with water, which insures a perfect

outflow and avoids any formation of air bubbles.

By using a stationary vat we avoid all the errors due to defective vats or to an imperfect cleaning. We do not need to know the exact thickness of the vat, since all curves of gauging are made with the same vat. All expenses for the purchase of optical vats are also avoided.

In sum, we offer an equipment which permits a rapid, precise and easy work with quantities of liquid of the order of 10 μl (0.01 ml).

All known quantitative determinations can be made at this scale with the usual methods. Furthermore, the proposed equipment does away with almost any washing and it is practically unbreakable. The laboratory space for these works is greatly reduced so that the installation of a quantitative analysis laboratory becomes possible in an automobile or in an airplane.

Technicians who have become used, for years, to the current methods have much more trouble to adapt themselves to these methods than people who have never worked in a laboratory. We have had excellent results with people who were totally without experience. It is largely for this reason that in countries like Iran and the Lebanon there is a lively interest for these methods.

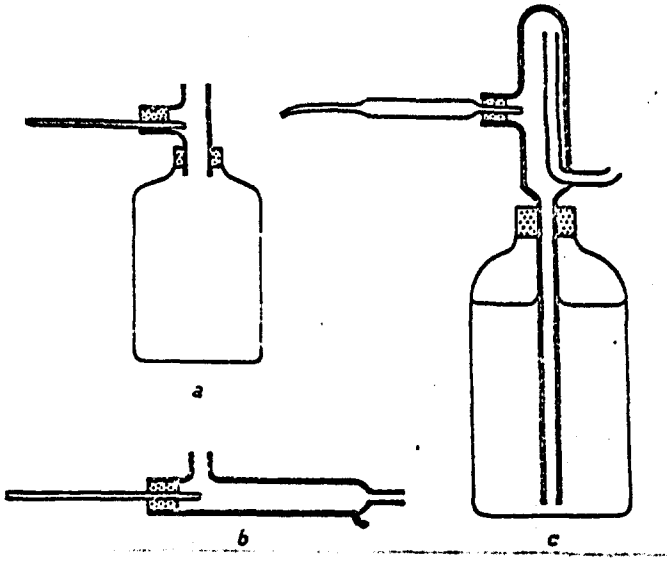

Fig. 1. - A-Pipette for hand-controlled samples; B - Pipette for samples controlled with the mouth, with the aid of a little tube; C - Pipette with tank for reagents.

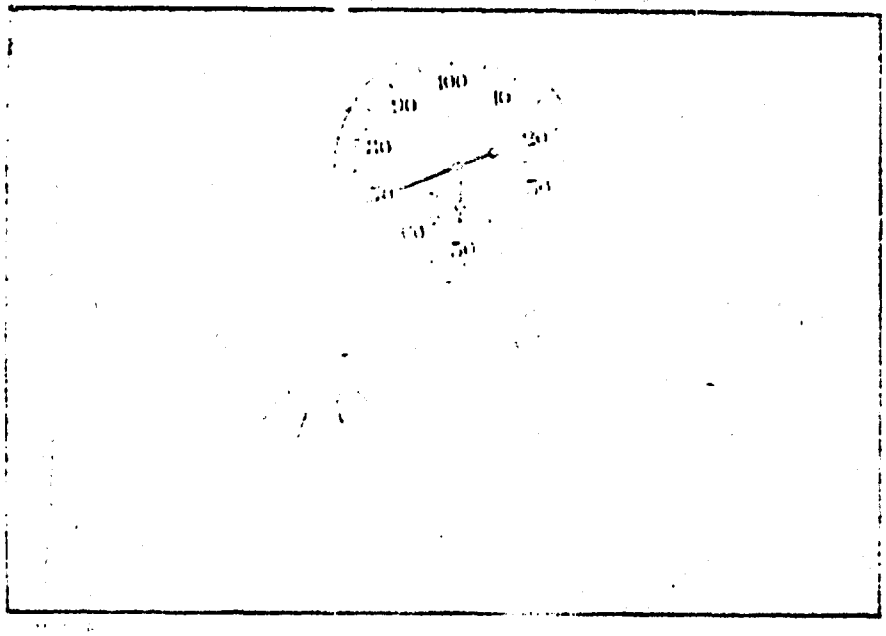

Fig. 2. - Ultramicro test-tube with automatic reduction to zero. 1 turn of the big needle corresponds to 3 μl.